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Physiological Regulation of Coccolith Polysaccharide Production by Phosphate Availability in the Coccolithophorid *Emiliania huxleyi*

Keisuke Kayano and Yoshihiro Shiraiwa*
Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, 305-8572 Japan

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Coccoliths of the coccolithophorid *Emiliania huxleyi* are calcified biomineral scales composed of calcium carbonate and coccolith polysaccharide (CPs). Coccolith production is regulated by inorganic phosphate (P_i) availability, but no information currently exists on how this process occurs. In this study CP was experimentally characterized by HPLC analysis as an acid polysaccharide of mannose, galacturonic acid, xylose and rhamnose. Both calcification (estimated from ^{45}Ca uptake) and CP production (estimated from uronic acid quantification) were stimulated under P_i-deficient conditions but strongly suppressed under P_i-sufficient conditions. When cells were transferred from P_i-sufficient to P_i-deficient conditions the production of neutral polysaccharides (NP)—storage glucans—ceased rapidly after a temporary increase in the presence of P_i, and CP production started to increase after P_i was almost depleted. Under P_i-sufficient conditions NP production increased, concomitant with stimulation of cell growth. Calcification increased gradually, but photosynthetic $^{14}CO_2$ fixation was reduced by almost 40% for 5 d of culture during P_i depletion. [^{14}C]CP production was maintained at almost constant, high levels under P_i-deficient conditions but gradually decreased under P_i-sufficient conditions in conjunction with cell growth. In contrast, [^{14}C]NP production increased about 3-fold under P_i-sufficient conditions for 3 d. The present study indicates that *E. huxleyi* switches the direction of carbon flow toward CP and NP production under P_i-deficient and P_i-sufficient conditions, respectively.

Keywords: Acid polysaccharide • Calcification • Carbon metabolism regulation • Coccolith polysaccharide • *Emiliania huxleyi* • Phosphate deficiency.

Abbreviations: ABEE, 4-aminobenzoic acid ethyl ester; AP, acid polysaccharide; BAS, bio-imaging analyzer system; CP, coccolith polysaccharide; IP, imaging plate; NP, neutral polysaccharide; TCA, trichloroacetic acid.

Introduction

Coccoliths are calcified scales produced by a group of marine haptophyte algae called coccolithophorids. Because coccoliths have highly elaborate species-specific structures, the methods used by cells to control crystal formation, both morphologically and biochemically, are of scientific interest. In addition, information relating to the mechanisms underlying this control is practical for the manufacture of new inorganic crystalline materials in biomimetic engineering (Cölfen 2003).

An acid polysaccharide (AP) called coccolith polysaccharide (CP) is the major organic component of coccoliths in one representative coccolithophorid, *Emiliania huxleyi* (Westbroek et al. 1973). The biochemical characteristics of CP include the ability to bind to calcium ions (de Jong et al. 1976) and to the surface of $CaCO_3$ crystals (Henriksen et al. 2004), as well as the ability to inhibit and modify in vitro $CaCO_3$ crystal formation (Borman et al. 1982 and Didymus et al. 1993, respectively). CP has a complex molecular structure consisting of >13 monosaccharides (Fichtinger-Schepman et al. 1981), and the steric configuration of carboxyl groups in galacturonic acid residues contributes to its range of functionalities (Borman et al. 1982). Relevant APs have also been found in three other species of coccolithophorids: *Pleurochrysis carterae* (Marsh et al. 1992), *Pleurochrysis haptonemofera* (Hirokawa et al. 2005, Ozaki et al. 2007) and

*Corresponding author: E-mail, emilhux@biol.tsukuba.ac.jp; Fax, +81-29-853-6614.

Plant Cell Physiol. 50(8): 1522–1531 (2009) doi:10.1093/pcp/pcp097, available online at www.pcp.oxfordjournals.org
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Gephyrocapsa oceanica (Ozaki et al. 2004). APs, including species-specific sugar compositions, are located in coccoliths and share common chemical features; they are likely to play an important role in the formation of highly elaborate species-specific coccoliths, but as yet there is no direct evidence that APs, and hence CPs, possess this function.

In addition, there are few studies reporting on the biosynthesis of AP. One study into the cytological localization of CP in *E. huxleyi* implied that it is produced in the Golgi body, transported to the coccolith-producing vesicle, and then deposited on the cell surface together with the coccolith (van Emburg et al. 1986). Such behavior was also observed in *P. carterae* (Marsh et al. 1992, Marsh 1996). However, the corresponding biosynthetic pathway and its metabolic regulation are unknown.

Environmental factors affecting coccolith formation, such as macronutrients (CO_2, P_i and NO_3^-), trace elements (Mg, Mn and Zn), temperature and salinity, have been studied in detail (Zondervan 2007). P_i limitation increases the number of coccoliths produced by *E. huxleyi* cells (Paasche 1998) and calcification is stimulated by low temperature signals (Sorrosa et al. 2005). P_i limitation is the primary signal triggering coccolith production, and low temperature is the secondary signal (Satoh et al. 2009). These results were based on the observation that coccolith production increased along with P_i deprivation, as indicated by the induction of alkaline phosphatase activity as a cellular response, and that the process proceeded even at the optimum temperature for growth (Satoh et al. 2009). However, how P_i deprivation regulates calcification is still unclear.

In the present study, we investigated the regulation of CP production by changing P_i availability in *E. huxleyi* cells using a radiotracer technique when giving $NaH^{14}CO_3$ as a substrate under P_i-sufficient and P_i-deficient conditions. In addition, ^{45}Ca radionuclide was used to monitor $CaCO_3$ crystal formation and to compare with data on [^{14}C]CP production in coccolith formation. We found that upon polysaccharide production carbon metabolism switches from storage polysaccharides, namely neutral polysaccharides (NPs), to CP production, and that $CaCO_3$ crystal formation and CP production are regulated simultaneously during coccolith formation when P_i availability decreases. These results lead to a better understanding of the regulation of coccolith production by *E. huxleyi* cells and the function of CP in vivo.

Results

Identification of CP

Polysaccharides were extracted with 5% trichloroacetic acid (TCA) from whole *E. huxleyi*, including coccoliths on the cell surface, to enable their characterization. The extracts were subjected to anion exchange chromatography after dialysis to obtain AP and NP. Following SDS–PAGE, AP was visualized as a strong band as a result of staining with both Stains-all and Alcian blue. This band was very similar to that of the polysaccharide extracted from isolated coccoliths of the *E. huxleyi* strain used in this study (**Fig. 1A**). There were also some minor positive bands in addition to the major one, which we consider to be derivatives of CP. Although the SDS–PAGE profiles of the AP from P_i-deficient and P_i-sufficient cells looked almost identical, the AP of P_i-deficient cells varied slightly from that of P_i-sufficient cells in mobility and also in terms of staining less intensely with Alcian blue, showing that the molecules contained more uronic acid residues. In addition, quantification of the polysaccharide content using a phenol–H_2SO_4 assay, followed by analysis of uronic acid content using a carbazol–H_2SO_4 assay, revealed that 4 µg of AP from P_i-deficient and P_i-sufficient cells contained 0.83 and 0.68 µg of uronic acid, respectively. CP isolated from the coccoliths of P_i-deficient cells was shown to contain more uronic acid (0.98 µg per 4 µg of total sugar components). Composition analysis of the AP by HPLC showed that it was composed of at least four sugars (mannose, xylose, rhamnose and galacturonic acid), which were previously reported as constitutive sugars of CP by Fichtinger-Schepman et al. (1981; **Fig. 1B**). These data clearly show that the AP from the *E. huxleyi* strain used in the present study was the same as the CP reported previously (Westbroek et al. 1973, de Jong et al. 1976). However, the NP was composed solely of glucose (**Fig. 1B**) and no positive bands were observed following SDS–PAGE and staining of gels with Stains-all or Alcian blue.

Change in CP content and calcification in whole cells during growth under various Pi concentrations

The growth of *E. huxleyi* cells was strongly suppressed following the limitation of P_i (**Fig. 2A**). In basal medium containing 28.7 µM P_i the linear growth of cells was maintained for 4 d; however, the growth slowed after about 5 d as a result of P_i depletion (**Figs. 2A, 3A**). These data show that the concentration of P_i as opposed to other nutrients was a limiting factor for *Emiliania* growth in culture. In contrast to algal growth, ^{45}Ca uptake by whole cells (a measure of calcification; Satoh et al. 2009) and uronic acid content detected using the carbazole–H_2SO_4 assay (a measure of the amount of CP) gradually increased under P_i-deficient conditions, whereas neither phenomenon was observed under P_i-sufficient conditions (**Fig. 2B, C**). A complete absence or reduction in calcified cells was consistently observed under microscopic examination (data not shown). It is possible that the amount of uronic acid determined might have been overestimated because CP contains many neutral sugar residues, which the carbazole–H_2SO_4 assay is also able to detect as a result of non-specific reactions (Bitter and Muir 1962). However, the data show that the rate of calcification, namely $^{45}CaCO_3$ production, far exceeds that of CP production

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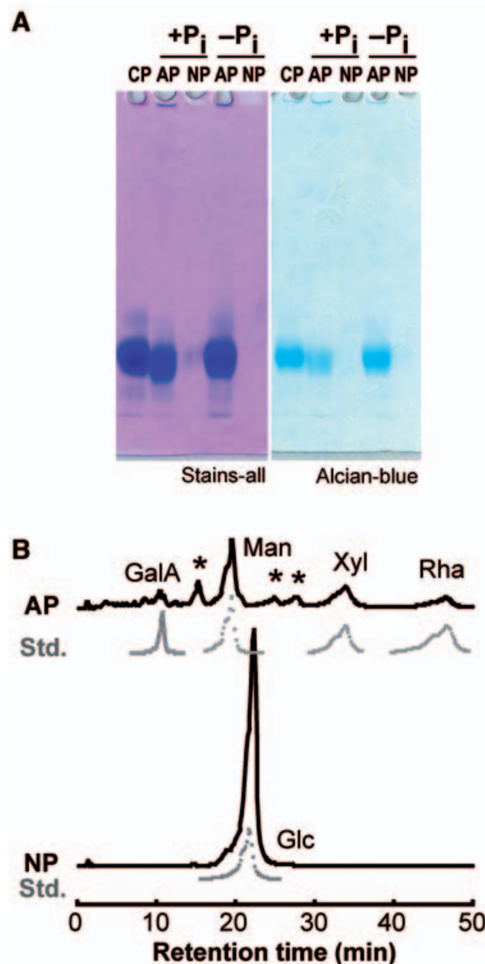

Fig. 1 Characterization of AP and NP isolated from *Emiliania huxleyi* cells grown in P_i-sufficient and P_i-deficient media. (A) SDS–PAGE patterns. A 4 µg aliquot of polysaccharide was loaded per lane, as described in Materials and Methods. The gel was stained with Stains-all and Alcian blue, separately. (B) Composition analysis of the AP and NP in A, from cells grown under P_i-deficient conditions, by HPLC. Peaks with asterisks (*) are not identified. CP refers to coccolith polysaccharides isolated from the coccoliths as a standard; AP, acid polysaccharide; NP, neutral polysaccharide; $+P_i$ and $-P_i$, P_i-sufficient and P_i-deficient cells, respectively; Std., standard compound.

(**Fig. 2D**). The data calculated per unit of cells show a clearer difference between both P_i conditions (**Fig. 2E, F**). These data imply that P_i limitation is a trigger for both calcification and CP production.

When CP and NP were determined separately after purification, we found that the amount of CP increased slowly for 3 d and rapidly thereafter (**Fig. 3**). In contrast, NP content increased immediately after the transfer from P_i-sufficient to P_i-deficient conditions and then stabilized at day 3. Although the reason for this increase is not known, it is clear that the increase in NP content occurred slightly before P_i depletion, and was followed by a significant increase in CP production (**Fig. 3B**). Both NP and CP content per cell remained very low in the presence of P_i (final concentration 28.7 µM; **Fig. 3C**), but this seems to have been caused by the marked increase in cell number due to cell division (**Fig. 3A, B**).

Change in calcification and CP production under various Pi conditions

After pre-culturing *E. huxleyi* cells for 4 d to deplete P_i in the medium, the culture suspension was divided in two. One half was maintained continuously in P_i-deficient medium, and the other was transferred to P_i-sufficient medium to which P_i was added periodically to avoid P_i limitation (**Fig. 4**). The algal cells grew well in the P_i-sufficient medium for 3 d and growth then plateaued, whereas no growth occurred in the P_i-deficient medium (**Fig. 4A**). Cells harvested at 2, 3 and 5 d were individually transferred to a glass reaction vessel and incubated separately with either 2 mM $NaH^{14}CO_3$ or 10 mM $^{45}CaCl_2$ for 8 h. After terminating the reaction by harvesting, we extracted CP and subjected it to SDS–PAGE as described in Materials and Methods. Radioactive bands of [^{14}C]CP were then visualized on the imaging plate (IP; **Fig. 4B**). The bio-imaging analyzer system (BAS) image analysis of SDS–PAGE profiles of ^{14}C-labeled CP showed only one major radioactive band (**Fig. 4B**), although other minor bands stained with Alcian blue were also observed (**Fig. 1A**). The radioactivity was determined as the IP response with a unit of photostimulated luminescence by bio-imaging analysis (**Fig. 4C**). The accumulation of [^{14}C]CP within both cells and the coccoliths increased during 2–8 h of incubation. [^{14}C]CP synthesis by P_i-deficient cells remained at high levels during 5 d of cultivation, whereas that by P_i-sufficient cells decreased markedly over this time period. ^{45}Ca uptake by P_i-deficient cells increased significantly with cultivation time, whereas that of P_i-sufficient cells remained at low levels (**Fig. 4C**). These results suggest that $^{45}CaCO_3$ crystal formation increases if accompanied by CP production under P_i-deficient conditions, although the extent of calcification exceeds that of CP production. The rate of total ^{14}C uptake by cells was 40–100 times higher than that of ^{45}Ca uptake, even though the $NaH^{14}CO_3$ concentration (2 mM) was five times lower than that of $^{45}CaCl_2$ (10 mM; **Fig. 4C**). Therefore, the amount of ^{14}C incorporation into $CaCO_3$, namely $Ca^{14}CO_3$ production, was not as significant as the amount of ^{14}C fixed by photosynthesis. Thus, the amount of ^{14}C inorganic carbon in the medium decreased to about half of the initial concentration at 8 h because of utilization by cells, whereas most added ^{45}Ca remained in the medium (**Fig. 4C**). In P_i-deficient cultures the total ^{14}C uptake per cell gradually decreased, whereas that in P_i-sufficient medium remained unchanged, although ^{14}C uptake per cell in P_i-deficient conditions was slightly greater than that under P_i-sufficient conditions (**Fig. 4C**). These results suggest that the regulation of carbon metabolism by P_i availability does not apply to the

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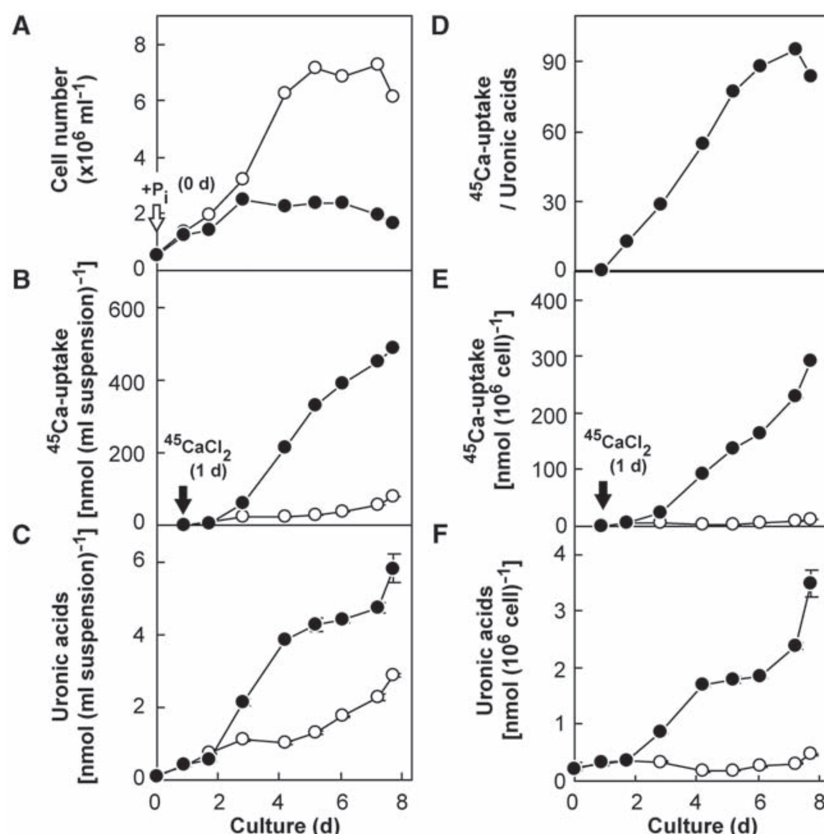

Fig. 2 Change in cell growth, calcification and acid polysaccharide content during the culture of a coccolithophorid, *Emiliania huxleyi*, under P_i-sufficient and P_i-deficient conditions. Cells pre-grown in a batch culture under aeration for 4 d until the cell density reached an OD_{750} of 0.5 were diluted 9-fold with fresh culture medium, with or without 28.7 µM P_i, at day 0 (white open arrows). $^{45}CaCl_2$ solution (final concentration, 9.5 mM; specific radioactivity, 0.24 MBq mmol^{-1}) was added on day 1 (black arrow). (A) Cell number; (B) and (E) ^{45}Ca uptake by cells; (C) and (F) uronic acid content; (D) ratio of ^{45}Ca uptake to the amount of uronic acid. The experiment was repeated three times in independent cultures, and a typical result is shown. Open and filled circles, transferred to P_i-sufficient ($+P_i$) and P_i-deficient ($-P_i$) media, respectively.

photosynthetic CO_2 fixation stage. It is possible that other regulatory mechanisms exist for the control of CP production by P_i. CP can be considered to be associated with calcification, but more detailed information on the function of CP itself and the relationship between amounts of CP and $CaCO_3$ produced is required.

Regulation of NP and CP production by Pi availability

To compare NP and CP synthesis, we pre-cultured cells in P_i-deficient medium for 2 d. The culture was then divided in two and incubated in P_i-sufficient or P_i-deficient medium for a further 3 d (**Fig. 5A**). ^{14}C uptake during 8 h of photosynthesis was approximately 25% higher in P_i-deficient cells than in P_i-sufficient cells (**Fig. 5B**). The accumulation of [^{14}C]NP increased 3-fold in P_i-sufficient cells, whereas no change was observed in P_i-deficient cells. In contrast, [^{14}C]CP production increased in both P_i-deficient and P_i-sufficient cells, although the extent of the increase was small in the latter compared with the former (**Fig. 5C**). These results suggest that the synthesis of storage polysaccharides such as NP is strongly stimulated by P_i.

In the ^{14}C labeling experiment with P_i-sufficient and P_i-deficient cells, almost half of the ^{14}C radioactivity (48 and 50%, respectively) was incorporated into the lipid and low molecular metabolite fraction, which probably included predominantly storage lipids such as alkenones, a few membrane lipids and low molecular metabolites (data not shown). Approximately 28 and 16%, respectively, of the total ^{14}C taken up by cells was incorporated into extracellular materials. In P_i-sufficient and P_i-deficient cells it was shown that ^{14}C was incorporated into proteins (20 and 26%, respectively) and low molecular polymers filtered through dialysis membrane (4.5 and 6.2%, respectively; data not shown). These results suggest that NP and CP production are closely regulated by P_i availability at the cellular level.

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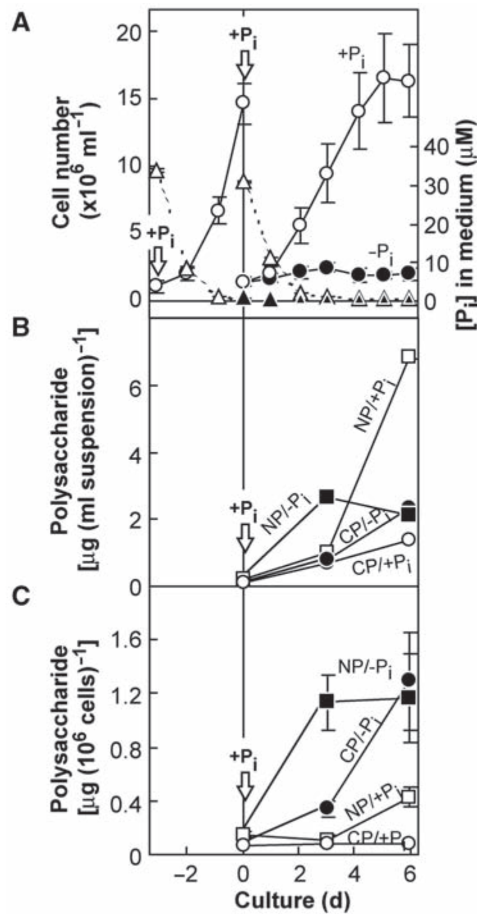

Fig. 3 Change in CP and NP content during the culture of *Emiliania huxleyi* cells under P_i-sufficient and P_i-deficient conditions. (A) Change in cell number (circles with solid line) and P_i concentration (triangles with dashed line) in the medium. Cells pre-grown in a batch culture under aeration were transferred to fresh media with or without 28.7 μM P_i, as indicated (white open arrows). (B) and (C) Change in CP (circles) and NP (squares) content. CP, coccolith polysaccharide; NP, neutral polysaccharide; open and filled symbols, transferred to P_i-sufficient (+P_i) and P_i-deficient (−P_i) media, respectively, at day 0.

Discussion

The presence of CP in coccoliths was first reported by Westbroek et al. (1973) and the complicated structure of the molecule was determined by Fichtinger-Schepman et al. (1981). However, the detailed physiological functions of CP and the metabolic process and regulation of CP production have yet to be elucidated. In this study, we found that the regulation of CP production is positively associated with calcification. Although P_i deficiency is known to trigger the calcification process in *E. huxleyi* cells (Paasche 1998, Satoh et al. 2009), here we present evidence that both CP production and $CaCO_3$ precipitation are regulated simultaneously by the change in P_i availability. Through a partial analysis of sugar components, we first confirmed that the AP extracted with 5% TCA from the *E. huxleyi* strain used in this study is the same as the polysaccharide isolated from the coccoliths (**Fig. 1**) and corresponds to the CP reported previously (Fichtinger-Schepman et al. 1981; **Fig. 1**). We then determined that both CP production (monitored by ^{14}C labeling) and calcification (monitored by ^{45}Ca uptake) are stimulated under P_i-deficient conditions, as compared with P_i-sufficient conditions (**Fig. 4B, C**).

As [^{14}C]CP production was maintained at almost constant, high levels for 5 d under P_i-deficient conditions (**Fig. 4C**), CP production could proceed almost linearly, as shown in **Fig. 2**. Evidence exists that calcification is strongly stimulated after 3 d (Satoh et al. 2009). This is compatible with the increase in the ratio of calcification to uronic acid content (**Fig. 2D**), and suggests that $CaCO_3$ production occurs at a higher rate than CP production, which proceeds in a constant manner. In addition, the synthesis of CP may occur continuously in cells because the amount of [^{14}C]CP in both the cells and coccoliths increased continuously for 8 h (**Fig. 4**). Thus, a substantial amount of CP—about a half of that synthesized—is likely to remain in the cell without being excreted with the coccoliths. However, the fate of the remainder of the CP with regard to degradation, metabolism to other molecules or reuse for coccolith production is unknown. According to cytological observations, polysaccharides in the coccolith-producing compartment that have not yet been experimentally identified as CP disappeared after the completion, but before the excretion of coccoliths (van der Wal et al. 1983). In *P. carterae*, which contains three kinds of AP (PS-1, PS-2 and PS-3), about 25% of PS-3 remained in cells even after 6 h of ^{14}C pulse–chase labeling, whereas most PS-1 and PS-2 was excreted with coccoliths in 2 h (Marsh 1996). Only PS-3 contains mannose, xylose and sulfate groups as components and is reported to be the same as the CP in *E. huxleyi* (Marsh et al. 1992). It should be noted that PS-3 was reported to be the main molecule controlling the specific morphology of coccoliths in a study using a natural variant that had lost the ability to produce PS-3 (Marsh et al. 2002).

Immunological quantification of CP showed that 0.44 μg (10^6 cells)$^{-1}$ and 0.56 μg (10^6 cells)$^{-1}$ of CP was excreted to the medium under nutrient-sufficient and -deficient conditions, respectively, for 6 d in a batch culture of *E. huxleyi* (Nanninga et al. 1996). These amounts are comparable with those of CP measured in this study, although the experimental conditions were different (**Fig. 3**). The determination of CP released into the medium is required to enable further understanding of CP metabolism.

NP containing only glucose, which was isolated by anion exchange chromatography, was also discovered in the present study. This appears to correspond to β-glucan, which was identified as an aqueous acid-soluble sugar and reported to be a cytosolic storage polysaccharide in *E. huxleyi*

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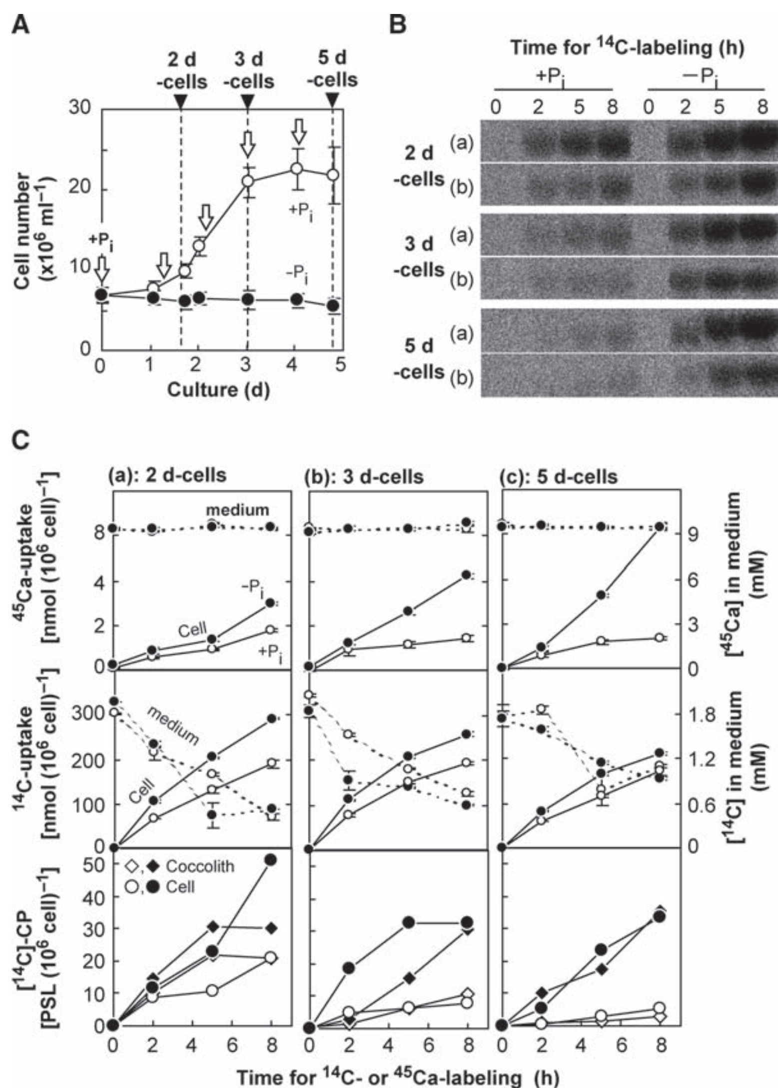

Fig. 4 Change in cell growth, calcification and CP production during the culture of *Emiliania huxleyi* cells under P_i-sufficient and P_i-deficient conditions. (A) Change in cell number. Cells were harvested by centrifugation from a batch culture maintained under aeration for 3 d and then transferred to fresh medium, with or without 28.7 µM P_i, at day 0 for a standing culture. To maintain a sufficient P_i concentration, we added 28.7 µM K_2HPO_4 every day as indicated by white arrows. At 2, 3 and 5 d (black arrowheads on top), part of the culture was subjected to ^{14}C and ^{45}Ca labeling using $NaH^{14}CO_3$ (final concentration, 2 mM; specific activity, 9.25 MBq mmol^{-1}) and $^{45}CaCl_2$ (final concentration, 9.5 mM; specific activity, 0.39 MBq mmol^{-1}) as substrates. (B) Bio-imaging analysis of radioactive bands of [^{14}C]CP isolated from whole cells including coccoliths (a), and naked cells from which coccoliths were removed artificially (b), on SDS–PAGE. (C) Time course of ^{45}Ca and ^{14}C uptake by cells and [^{14}C] CP produced in the cellular and coccolith fractions during 8 h radiolabeling. CP, coccolith polysaccharide; PSL, photostimulated luminescence.

(Vårum et al. 1986, Raven 2005). However, the amount of polysaccharide determined as NP in this study (**Figs. 3, 5**) was smaller than that of β-glucan, which was reported to be equivalent to 16% (w/w) of the dry cell weight (Vårum et al. 1986), because the small glucan molecule was fractioned as a low molecular weight polymer following dialysis treatment. Vårum et al. (1986) reported that the average degree of polymerization of β-glucan was 106, corresponding to the average molecular weight of approximately 17,000 Da in *E. huxleyi*. The small glucan molecule equated to 4.5–6.2% of the total ^{14}C incorporation, irrespective of P_i availability. These compounds may include intermediates of the NP metabolic pathway. Thus, not all β-glucan was determined to be NP in the present study.

NP production seems to be stimulated under P_i-sufficient conditions (**Figs. 3, 5**). The consumption or metabolism of NP may also be stimulated in P_i-sufficient cells because the cells actively divide for growth, as shown in **Fig. 3A**. NP

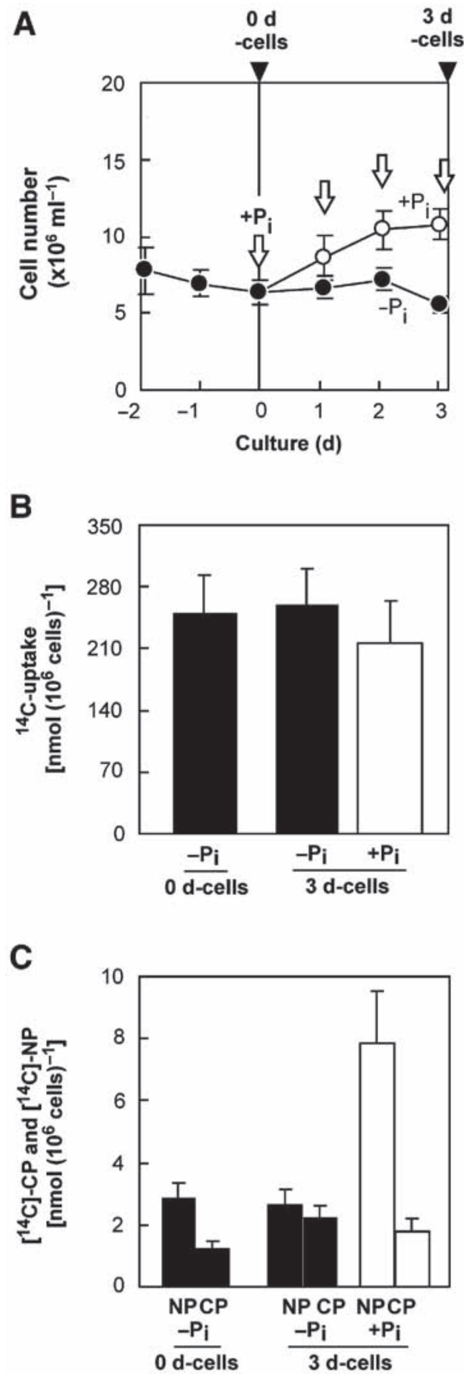

Fig. 5 ^{14}C labeling patterns of CP and NP in *Emiliania huxleyi* cells grown under P_i-sufficient and P_i-deficient conditions. (A) Algal growth. Cells harvested by centrifugation from the pre-culture were transferred to fresh medium without P_i for the first 2 d (−2 to 0 d). At day 0 the culture was divided in two; one half was maintained as before, whereas the other was maintained in P_i-sufficient medium by periodically adding P_i as K_2HPO_4 corresponding to 28.7 µmol l^{-1} every day, as indicated by white arrows. At days 0 and 3 (black arrowheads on top), part of the culture was incubated with $NaH^{14}CO_3$ (final concentration, 2 mM; specific activity, 9.25 MBq mmol^{-1}) for 8 h. (B) ^{14}C uptake by cells for 8 h. (C) Change in the amount of cellular [^{14}C]CP and [^{14}C]NP. The polysaccharides were isolated from naked cells from which coccoliths on the cell surface were removed by EDTA treatment, as described in Materials and Methods. CP, coccolith polysaccharide; NP, neutral polysaccharide.

production was stimulated about 3-fold under P_i-sufficient conditions (**Fig. 5**), suggesting that the cells actively synthesize storage polysaccharides, resulting in an increase in growth. In contrast, NP production remained constantly low for 3 d of cultivation under P_i-deficient conditions, resulting in a strong suppression of cell growth (**Fig. 5**).

In general, two types of response to P_i deficiency are observed in higher plants and algae: a primary response related to the efficient acquisition of P_i (e.g. induction of phosphatase and P_i transporters) and subsequent metabolic changes (e.g. accumulation of storage polysaccharides; Nilsson et al. 2007). With regard to the production of storage polysaccharides, several nucleotide diphosphate sugar pyrophosphorylases, which provide substrates for polysaccharide synthesis as key enzymes, are stimulated by P_i deficiency (Collén et al. 2004, Nilsson et al. 2007). In *E. huxleyi*, the induction of alkaline phosphatase activity occurred before calcification under conditions of P_i deficiency (Satoh et al. 2009), suggesting that calcification requires a trigger after receiving a P_i-deficient signal. In contrast, CP production does not seem to be directly regulated by P_i itself because CP production in 2-day-old cells not fully acclimated to P_i-sufficient conditions showed no substantial difference, irrespective of P_i condition, and required 5 d to acclimatize to complete suppression of [^{14}C]CP production (**Fig. 4C**, a). Moreover, another coccolithophorid, *Pleurochrysis*, continuously produces coccoliths independently of P_i availability, even as it synthesizes the CP-like AP, PS-3, and accumulates β-glucan as a storage polysaccharide (van der Wal et al. 1987, Hirokawa et al. 2008).

A model of the regulatory profile of CP and NP production by P_i availability in *E. huxleyi* is shown in **Fig. 6**. CP production is stimulated under P_i-deficient conditions and suppressed under P_i-sufficient conditions. In contrast, NP production is activated under P_i-sufficient conditions. The present study suggests that the adverse regulation of the production of CP and NP is due to the polysaccharides' different localization and function. Understanding the metabolic relationship between CP and coccolith production, as regulated by P_i availability, requires further detailed study of the metabolic pathways for and gene regulation of CP and NP. In addition, a comparative study of CP production in coccolithophorids that possess inducible and constitutive processes for coccolith production (e.g. *E. huxleyi* and *P. haptonemofera*, respectively) will be very useful for understanding

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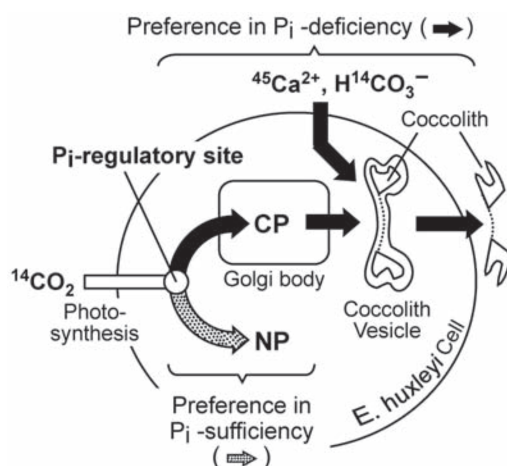

Fig. 6 Model of the metabolic regulation of coccolith and neutral polysaccharide production and calcification by P_i availability in *Emiliania huxleyi* cells.

the physiological and molecular regulation of coccolith production.

Materials and Methods

Organism details and culture conditions

The strain of coccolithophorid *E. huxleyi* (Lohman) Hay & Mohler (Haptophyceae; NIES 837) used in the present study was collected by Dr. I. Inouye of the University of Tsukuba in the South Pacific Ocean in 1990. Cells were grown in natural seawater for stock culture and in artificial seawater (Marine Art SF; produced by Tomita Seiyaku Co., Ltd., Tokushima, formerly distributed by Senju Pharmaceutical Co., Osaka, Japan, and recently by Osaka Yakken Co. Ltd., Osaka, Japan) enriched with Erd-Schreiber's medium containing 10 nM sodium selenite instead of soil extracts (Danbara and Shiraiwa 1999). Cells were maintained under constant illumination at 100 µmol m^{-2} s^{-1} and 20°C (standard condition). Erd-Schreiber's medium contains 28.7 µM K_2HPO_4, but Marine Art SF does not. To produce the phosphate-deficient condition, K_2HPO_4 was removed from the medium.

Extraction of polysaccharides and subsequent fractionation of CP and NP

Polysaccharides were extracted from *E. huxleyi* cells using TCA according to de Jong et al. (1979). Briefly, cells were harvested by centrifugation and stored at −80°C prior to ultrasonic lysis at 4°C using 5% (w/v) TCA. The homogenates were centrifuged at 15,000 × *g* for 1 h, and the supernatant was either analyzed for uronic acid content or used for CP or NP purification. For the purification of CP, the supernatant was dialyzed with dialysis membrane (molecular weight cutoff 12–14 kDa; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the solvent changed to 20 mM Tris–HCl (pH 8.0). The sample was then subjected to anion exchange liquid chromatography using a HiTrap DEAE FF (GE Healthcare UK Ltd., Buckinghamshire, UK). The fractions eluted with the same buffer containing 0.5 M NaCl were collected as the AP, whereas the fractions that flowed through the column were collected as the NP. Each polysaccharide fraction was dialyzed against deionized water and either stored or lyophilized for further analysis.

Assays

Uronic acid content was estimated using a carbazole–H_2SO_4 assay (Bitter and Muir 1962). For calibration, 0–90 µg ml^{-1} glucuronic acid (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) was used as the standard. The amount of polysaccharides was estimated using a phenol–H_2SO_4 assay (Hodge and Hofreiter 1962). For calibration, 0–90 µg ml^{-1} glucose was used as the standard. Inorganic phosphate concentration in the medium was determined using the molybdenum blue method (Murphy and Riley 1962). To obtain a cell-free medium, cells were removed by centrifugation at 15,000×*g* for 2 min, twice.

Electrophoresis of polysaccharides

To characterize the molecular properties, namely electrostatic mobility and specific staining, of the polysaccharides, we subjected isolated CP, AP and NP to SDS–PAGE. The amount of polysaccharides applied to each lane was 4 µg, corresponding to the glucose used as a standard in the phenol–H_2SO_4 assay. Notably, APs from P_i-sufficient and P_i-deficient cells were estimated to contain 0.68 and 0.83 µg uronic acid, respectively, corresponding to glucuronic acid used as a standard in the carbazol–H_2SO_4 assay. In other experiements, CP isolated from only the coccoliths was used as a standard and it was found that 4 µg of CP contained 0.98 µg of uronic acid (see above). After electrophoresis, the gels were stained with Stains-all or Alcian blue. Alcian blue staining was carried out with 7.5% (v/v) acetic acid. No positive bands were detected as a result of Coomassie brilliant blue staining in any of the samples. For standard CP, coccoliths were isolated from cells using the method of de Jong et al. (1976), except that 80% (w/v) sucrose was used instead of 50% Ludox in the coccolith purification process.

Analysis of polysaccharide composition

CP and NP isolated from P_i-deficient cells were hydrolyzed with 4 M trifluoroacetic acid for 3 h at 100°C. Hydrolyzed samples were modified with 4-aminobenzoic acid ethyl ester (ABEE) according to the manufacturer's instructions (Seikagaku Co., Tokyo, Japan). The ABEE-modified monosaccharides were analyzed by HPLC using Honenpak C-18 (Seikagaku Co.). Each monosaccharide was identified with the standard sugars, individually prepared and subjected to HPLC in the same way.

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^{45}Ca uptake

The amount of coccolith production was measured based on Satoh et al. (2009) by determining the incorporation of ^{45}Ca into the cells, based on evidence that >95% of ^{45}Ca was taken up by whole cells, located in the coccoliths. An adequate amount of $^{45}CaCl_2$ (Perkin-Elmer, Inc., Waltham, MA, USA) was added to the culture (see respective figures and legends). At intervals, 0.2–1 ml of culture was harvested, and the cells were separated from the medium by centrifugation at 15,000×*g* for 2 min after the addition of 0.01% (w/v) Tween-20. The pellet was washed twice with fresh medium, and radioactivity in the pellet (cells) and supernatant (medium) was determined using a liquid scintillation counter (LS 5000TD; Beckman Coulter, Inc., Fullerton, CA, USA) with a scintillation cocktail (Hionic-Fluor; Perkin-Elmer).

Determination of ^{14}C incorporated into CP and NP

The initial rate of CP production was estimated using a ^{14}C labeling technique. An adequate amount of $NaH^{14}CO_3$ (GE Healthcare) was added to the pre-culture (see respective figures and legends). At intervals, an aliquot of cell suspension was harvested for further analysis. After the addition of 0.01% (w/v) Tween-20 to the suspension for smooth precipitation, the cells were obtained by centrifugation at 15,000×*g* for 2 min. The supernatant was treated with 1 M NaOH to avoid volatilization of CO_2 and the pellet was washed twice with fresh medium. Radioactivity in the pellet (cells) and supernatant (medium) was determined using a liquid scintillation counter.

For analysis of ^{14}C-labeled CP, a 5% TCA extract was prepared from whole cells, as described above. To determine CP located inside the cells, we treated part of the sample with a solution containing 0.1 M EDTA and 60 mM Tris–HCl (pH 8.0) to dissolve the extracellular coccoliths attached to the cell surface prior to 5% TCA extraction. The TCA extracts were washed and concentrated by co-precipitation with 20 µg of the CP independently prepared without ^{14}C labeling, using 83% acetone. The pellet was washed with 83% acetone again and then subjected to SDS–PAGE after dissolving in distilled water. Electrophoresis samples were normalized based on the harvested cell number (about 10^7 cells). The gels were stained with Alcian blue and then dried under a heated vacuum using a gel dryer. Radioactive bands corresponding to CP were quantified as the IP response with a unit of photostimulated luminescence using a BAS (BAS-1800II; Fujifilm, Tokyo, Japan).

^{14}C-labeled NP was purified using the following procedure. Cells were treated with a solution containing 0.1 M EDTA and 60 mM Tris–HCl (pH 8.0) to remove coccoliths and extracellular materials (extracellular materials fraction), then disrupted with 5% TCA. Cell disruption was repeated twice and the analyses were combined. Further extraction of the pellet with 5% TCA at 60°C resulted in approximately 60% of ^{14}C radioactivity remaining in the pellet, although the levels of radioactivity were not high. The pellet was washed with 83% acetone (lipid and low molecular metabolite fraction 1), solubilized with 0.1 N NaOH at 60°C and then precipitated again with 5% TCA at 60°C (protein fraction). The supernatant was dialyzed through a dialysis membrane (Wako Pure Chemical Industries, Ltd.) to obtain compounds with molecular weights >12–14 kDa (low molecular polymers fraction 1). What remained in the dialysis tube was an NP fraction that contained sugars (detectable by the phenol–H_2SO_4 assay) but no uronic acid or CP (detectable by the carbazole–H_2SO_4 assay or by SDS–PAGE followed by Alcian blue staining and bio-imaging analysis). Meanwhile, the polysaccharide fraction extracted with 5% TCA was co-precipitated with 20 µg of CP using 83% acetone to obtain lipids and low molecular metabolite fraction 2 in the supernatant. The pellet was dissolved in, and dialyzed against, 20 mM Tris–HCl (pH 8.0) to obtain low molecular weight polymers fraction 2 in the dialysate. The CP and NP fractions were separated using anion exchange chromatography as described above. The radioactivity in each fraction was determined using a liquid scintillation counter. No significant effect of chemical quenching was observed in the scintillation cocktail Hionic-Fluor (Perkin-Elmer, Inc.).

Funding

The Salt Science Foundation (0025 to Y.S. in 2000); the Japan Science Society (Sasakawa Scientific Research Grant 18-309M to K.K.).

Acknowledgments

We thank Drs. Y. Jigami and T. Oka of the National Institute of Advanced Industrial Science and Technology and the Faculty of Biotechnology and Life Science, Sojo University (T.O. at present), for their kind help with the sugar composition analysis and their helpful suggestions on polysaccharide metabolism. We also thank Dr. I. Suzuki of our laboratory for valuable support with the experiments, and the staff at the Radioisotope Center of the University of Tsukuba for their kind help with radioisotope experiments.

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(Received March 4, 2009; Accepted June 25, 2009)

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